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A quartz crystal biosensor for measurement in liquids.
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Uttenthaler E, Kosslinger C, Drost S.
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Biosens Bioelectron. 1998 Dec 1;13(12):1279-86.

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920035

Article title	A quartz crystal biosensor for measurement in liquids
Article identifier	095656639200059K
Authors	Kosslinger_C Drost_S Aberl_F Wolf_H Koch_S Woias_P
Journal title	Biosensors and Bioelectronics
ISSN	0956-5663
Publisher	Elsevier UK
Year of publication	1992
Volume	7
Issue	6
Supplement	0
Page range	397-404
Number of pages	8
User name	Adonis
Cost centre	
PCC	$22.00
Date and time	Tuesday, September 09, 2003 4:29:25 AM

Biosensors & Bioelectronics 7 (1992) 397–404

A quartz crystal biosensor for measurement in liquids

Conrad Kößlinger[a], Stephan Drost[a], Franz Aberl[b], Hans Wolf[b], Sabine Koch[c] & Peter Woias[c]

[a]Fraunhofer-Institut für Festkörpertechnologie, Paul-Gerhardt-Allee 42, D-8000 München 60, Germany
[b]Institut für Molekulare und Tumorvirologie am Max von Pettenkofer-Institut, Ludwig-Maximilians-Universität, München, Germany
[c]Lehrstuhl für Integrierte Schaltungen, Technische Universität München, Germany

(Received 17 May 1990; revised version received 3 October 1991; accepted 31 October 1991)

Abstract: The detection of anti-human immunodefficiency virus (HIV) antibodies by means of synthetic HIV peptide immobilized on a piezoelectric quartz sensor is demonstrated. The measurement set-up consists of an oscillator circuit, a suitably modified AT-cut thickness-shear-mode quartz crystal with gold electrodes, which is housed in a special reaction vessel, and a computer-controlled frequency counter for the registration of the measured frequency values. The quartz crystal is adapted for a steady operation in liquids at a frequency of 20 MHz. In phosphate-buffered saline solution the oscillator reaches a stability of about 0·5 Hz within a few seconds, of about 2 Hz within 10 min and about 30 Hz within 1 h. The frequency shift due to the adsorption of various proteins to the uncoated sensor surface has been investigated. It can be shown that a stable adsorptive binding of proteins to an oscillating gold surface is feasible and can be used for the immobilization of a receptor layer (e.g. HIV peptide). Specific binding of the anti-HIV monoclonal antibody to the HIV peptide immobilized on the quartz sensor is demonstrated. Control experiments show, however, additional unspecific binding. According to the experiments, the Sauerbrey formula gives a sufficiently accurate value for the decrease of the resonant frequency due to adsorption or binding of macromolecular proteins on the quartz crystal surface.

Keywords: piezoelectric crystal, biosensor, immunosensor, anti-HIV antibody.

INTRODUCTION

The principle of microbalance can be used to detect minute amounts of biological or chemical substances via mass accumulation; this therefore represents a promising transducing mechanism for immuno-biosensors. It is also possible to observe binding reactions of proteins at surfaces *in situ*. This paper focuses on microbalances based on piezoelectric crystals, where a decrease of the resonant frequency can be correlated to accumulated mass.

An immunosensor that uses immobilized antigens for the detection of antibodies or vice versa can be applied in the following fields:

clinical diagnostics (e.g. human immunodeficiency virus (HIV) test);

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environmental control (e.g. detection of micro-organisms in water);
- food industry (e.g. detection of toxic substances).

Compared to conventional measuring systems the advantages are short response times, the detection of the immunoreaction without additive reagents, and a direct conversion of mass accumulation into a frequency shift which represents an exactly measurable electrical signal.

Some recent publications show the possibility of direct immunosensing by bulk acoustic wave transducers (Davis & Leary, 1989; Muramatsu *et al.*, 1987, 1989; Thompson *et al.*, 1986). In contrast to these experiments, this paper describes investigations of an experimental procedure without a flow-through cell and with an antigen/antibody system which in the future might possess practical relevance for HIV research and HIV diagnostics.

The experiments demonstrate the effects of different protein-binding mechanisms to the oscillating sensor surface when measuring continuously in liquids.

The fundamental feasibility of an immunosensor based on a piezoelectric crystal is to be demonstrated. As a biological model system synthetic HIV peptide from the core shell of the HIV virus (p24) was used for the detection of the respective monoclonal antibody (Wolf *et al.*, 1990; Modrow & Wolf, 1990).

MEASUREMENT SET-UP

Figure 1 shows the measurement set-up, which was used for all experiments. The decisive difference from other measurement set-ups, described for example by Thompson *et al.* (1986), is the reaction vessel, which is not a flow-through cell. Thus the measurement procedure also differs from that described by Thompson *et al.* (1986) or by Muramatsu *et al.* (1987).

The reaction vessel contains an AT-cut quartz, operating at 20 MHz. It was mounted at the bottom of the reaction vessel, exposing only one electrode to the sample solution. The reaction vessel was made of stainless steel. The electrodes consisted of gold-plated nickel and were fabricated by Quarzkeramik GmbH, Stockdorf, Germany. The electrical feedback loop was located very close to the sensor quartz. The resonant frequency of the oscillator was determined by a computer-controlled frequency counter and was additionally monitored with a chart-recorder.

In order to detect correlations between variations of temperature and changes of frequency, the temperature was measured by a temperature-dependent platinum resistor (PT 100). The resistance was measured by a computer-controlled ohmmeter.

The reaction vessel and the oscillator circuit were placed in a chamber to protect the measurement against thermal, mechanical and electrical influences.

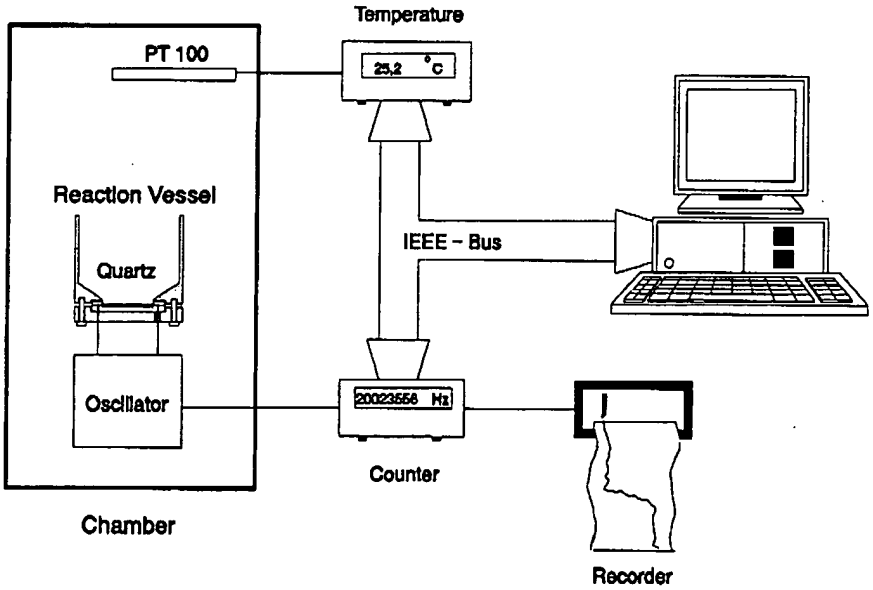

Fig. 1. Measurement set-up.

GENERAL FEATURES OF THE MEASUREMENT SYSTEM

The following parameters are essential for the characterization of sensor performance: detection limits, response times and mass sensitivities (i.e. the frequency response due to the mass change at the quartz surface, or, more generally, due to changes of the gold/water interface conditions like viscosity, density and sound velocity of surface layer).

The most important theoretical considerations dealing with sensitivity were published by Sauerbrey (1959), Chi-shun Lu (1975), and Kanazawa & Gordon (1985).

Kanazawa found the most extensive relation between change of frequency and viscoelastic properties at the surface of the quartz crystal. Lu considered the elastic properties of thick multilayers, whereas Sauerbrey's formula deals only with thin solid films on the quartz crystal surface. The greatest advantage of the Sauerbrey formula is that all parameters are easily accessible and the change of the resonant frequency only depends on the surface mass density (Sauerbrey, 1959):

$$\Delta f = S\Delta m$$

and

$$S = -2f^2/Z_p$$

where Δf = frequency shift, Δm = change of mass per area, S = mass sensitivity, Z_p = acoustical impedance, and f = resonant frequency.

With $Z_p = 8{\cdot}85 \times 10^{14}$ ng Hz cm^{-2} and a resonant frequency of 20 MHz the resulting sensitivity can be calculated as $S = 0{\cdot}904$ Hz ng^{-1} cm^2. In other words, a mass accumulation of 1 ng cm^{-2} causes a frequency shift of about 1 Hz.

However, this formula does not consider any viscoelastic effects, which might occur in liquids while binding reactions at the surface take place. Nevertheless, the Sauerbrey formula gives good values for estimations, as shown by the experiments described below. Viscoelastic effects do not play an important role for the performed experiments.

A short theoretical calculation for kinetics of immunochemistry at surfaces, which determines reaction times and frequency response times, was given by Thompson *et al.* (1987), who gives a solution of the Fick equation. There, a reaction time of 0·5 s for a coverage of 50% of the surface corresponds to an antibody concentration of 10^{-6} M. Lower antibody concentrations would cause lower rates of antigen/antibody formation. These calculations treat only boundary conditions which can be formalized. The procedure of carrying out experiments can be decisive for the response times. In this paper the resulting response times were in the range of a few minutes.

The detection limits depend on long-term stability, sensitivity, response time and the time that can be allowed for the measurement. Therefore no exact value for detection limits can be given. A value which is of some interest for detection limits is the minimum amount of mass at the quartz crystal surface, which causes a significant drop in frequency. For these considerations we assume a sensitivity of 1 Hz ng^{-1} cm^{-2}. The drop in frequency should be twice as much as the long-term fluctuations of frequency. These long-term fluctuations are likely caused by viscosity effects at the water-quartz interface, in contrast to the short time fluctuations, which are much smaller and caused by noise of the oscillator circuit. The used oscillator circuit with a 20 MHz quartz reveals ±0·5 Hz variation for observation intervals of 4 s, which is the detection limit of the used counter. The long-term stability over 1 h is about 30 Hz. With a required frequency drop of about 60 Hz, the above assumed sensitivity and an electrode area of about 0·05 cm^2 (2·5 mm diameter), a minimum amount of mass of about 3 ng is needed. But this value is theoretical and it is not the subject of these experiments to reach this boundary.

Another important fact is the influence of temperature effects. Quartz crystals with exact AT-cut have very good temperature stability at room temperature in air or in a vacuum. The temperature profile worsens dramatically when the quality factor of the quartz crystal falls from 40 000 in air to 5000 in water. This causes a temperature profile of 100 Hz K^{-1}. However, parallel measurement of temperature and frequency in water showed negligible temperature fluctuations. Nevertheless the temperature dependence has to be improved for better long-term stability over hours and days.

EXPERIMENTS

The following preparatory procedure was used for all experiments. First the mounted quartz

crystal was cleaned with hydrogen chloride (pH 3) and rinsed with chloroform. The reaction vessel was filled with 5 ml of phosphate-buffered saline (PBS) of pH 7·4. Then, a small amount of relatively highly concentrated sample solution was added. All experiments were performed without stirring. Rinsing was only used after the immobilization of the antigens. Owing to these procedures and the shape of the reaction vessel, we expected, after adding the sample solution, an inhomogenous distribution of proteins with a much higher concentration at the quartz electrode, which also should cause fast response times. These higher concentrations at the gold electrode were obviously caused by sedimentation. On the other hand, it was also expected that unspecific binding reactions should be promoted by this procedure. Furthermore, it was supposed that after reaching equilibrium the surface was nearly saturated. This was proved by the fact that further addition of proteins did not decrease the resonant frequency any more. Although boundary conditions were not defined as clearly as in a flow-through cell, the experiments showed positive results beyond doubt.

Each quartz crystal was used once to obtain the same starting conditions. Experiments to regenerate the immunosorbent surfaces have not been carried out owing to the aim of getting a disposable sensor for fast tests. Such a sensor can be used, for instance, in clinical tests as an alternative method to indirect immunological tests like ELISA.

The curves shown in Figs 2–5 are single measurements representing typical coarses of each type of measurement. The noise was smoothed out. Table 1 compares the measured values for the adsorption of proteins with the calculated data from the Sauerbrey equation. The surface particle density is given assuming a cube-shaped protein and a maximum package density. The frequency shift data are a summary of experiments using five different quartz crystals for every class of experiment, represented by a row in Table 1. The value of the frequency shift for a single measurement was rounded to 50 Hz

TABLE 1 Comparison between measured and expected frequency shift due to reaction of proteins with the surface on condition of maximum packed surface layer

Substance	Mol. wt ($\times 10^3$)	Surface particle density (cm^{-2})	Surface mass density ($g\,cm^{-2}$)	Expected shift of frequency (Hz)	Measured shift of frequency (mean value in Hz)	Standard deviation (Hz)	RDS (%)
BSA	65	$7{\cdot}0 \times 10^{12}$	$7{\cdot}7 \times 10^{-7}$	770	340	65	19
Anti-HIV peptide antibody	150	$4{\cdot}0 \times 10^{12}$	$1{\cdot}0 \times 10^{-6}$	1000	890	178	20
Anti-EBV antibody	150	$4{\cdot}0 \times 10^{12}$	$1{\cdot}0 \times 10^{-6}$	1000	920	172	19
Enzyme-labelled antibody	600	$1{\cdot}6 \times 10^{12}$	$1{\cdot}6 \times 10^{-6}$	1600	1560	318	20
Specific reaction of anti-HIV peptide antibody with HIV antigens	150	$4{\cdot}0 \times 10^{12}$	$1{\cdot}0 \times 10^{-6}$	1000	1000	146	15
Unspecific reaction of anti-EBV antibody with HIV antigens	150	0	0	0	320	152	48

because of noise. Standard deviation and relative standard deviation (RSD) were also calculated.

Adsorptive binding of different proteins

Figure 2 shows the measured frequency shift due to adsorbed bovine serum albumin (BSA). In the first step (indicated by arrow 1) 20 μl of BSA with a concentration of 1 mg ml^{-1} was added to a sample volume of 5 ml PBS (pH 7·4). In the second step (indicated by arrow 2) the same amount BSA was added again after 2 min, before equilibrium was reached. The quartz was previously cleaned with hydrogen chloride (pH 3) and rinsed with chloroform.

The reaction velocity was increased significantly by doubling BSA concentration. The saturation value for the frequency shift in this measurement was about 350 Hz for a total amount of 20 μg BSA in 5 ml PBS and was reached after 6 min.

Figure 3 gives a plot of the adsorption curves for various proteins, such as BSA (1), antibodies (2) and antibodies labelled with urease (3). The figure combines three separate measurements. The three curves were drawn shifted to each other for clarity. The amounts of protein added to 5 ml PBS were: for BSA 20 μl of a concentration of 1 mg ml^{-1}; for the antibodies, 5 μl of a concentration of 1 mg ml^{-1}; and for the enzyme-labelled antibodies, 5 μl of a concentration of 1 mg ml^{-1}.

Curve 2 represents a set of measurements with

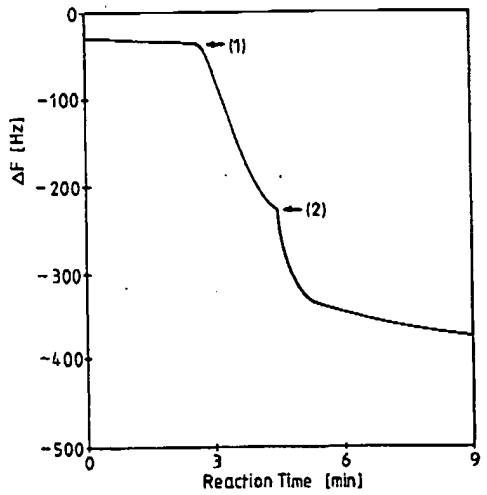

Fig. 2. Adsorption of BSA at the gold electrode. Five microlitres of BSA at a concentration of 10 μg ml^{-1} were added twice to a sample volume of 5 ml PBS (indicated by the two arrows).

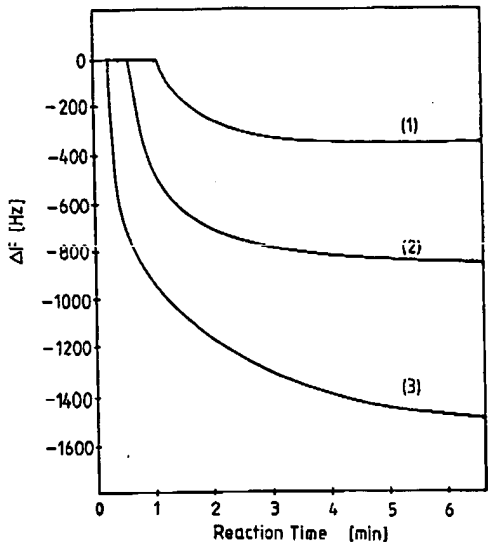

Fig. 3. Frequency response for adsorption at gold electrodes of BSA (1), antibodies (2) and enzyme-labelled antibodies (3). The figure combines three separate measurements. The three curves were drawn shifted to each other for more clarity. The amount of protein added to 5 ml PBS were: for BSA 20 μl of a concentration of 1 mg ml^{-1}; for the antibodies, 5 μl of a concentration of 1 mg ml^{-1}; for the enzyme-labelled antibodies, 5 μl of a concentration of 1 mg ml^{-1}.

two types of antibodies which show the same behaviour for adsorption at the gold electrode. The two antibody types were monoclonal antibodies purified by ammonium sulphate precipitation and specific to:

- a synthetically produced peptide of the core shell of the HIV virus (Wolf *et al.*, 1990; Modrow & Wolf, 1990);
- the Epstein-Barr virus (EBV).

The precipitates were not further purified. The enzyme-labelled antibody was an FC antibody, specific to the FC part of antibodies. According to higher-frequency drops, this FC antibody was labelled by urease.

Comparison between calculated and measured values in Table 1 shows generally the same tendency but the standard deviation is relatively high. The RSD is about 20%. The high RSD is probably caused by insufficiently reproducible experimental conditions. The measured data are below the expected maximum values.

As these measurements show a very stable binding of proteins to the electrode surface by mere adsorption, for further experiments an adsorptive immobilization of the antigen was

chosen. The effectivity of this immobilization was controlled with ELISA and found to be sufficiently high.

Binding of anti-HIV peptide antibody to adsorbed HIV peptide

The adsorptive immobilization of HIV peptide was performed according to the following procedure:

(1) Cleaning of the gold electrode with chloroform.
(2) Dissolving the peptide in phosphate buffer (pH 7,0·05 M) at a concentration of 10 μg ml^{-1}.
(3) Fifty microlitres of the above solution onto the quartz dropping; reaction allowed for 18 h at a temperature of 37°C.
(4) Rinse with PBS-Tween.

After immobilization of the peptide remaining unspecific binding sites were saturated with gelatine and once again the sensor was rinsed with PBS. For the measurement the reaction vessel (see Fig. 1) was filled with 5 ml PBS and 5 μl of anti-HIV antibody were added, corresponding to a dilution of 1:1000. For these preliminary measurements a precipitate of monoclonal antibodies was used, which contained about 1 mg ml^{-1} antibody. An exact value cannot be given, as the precipitate was not purified by affinity chromatography.

Figure 4 shows the response of the piezoelectric sensor to the addition of anti-HIV antibody (indicated by an arrow). The saturation value of the frequency shift (1000 Hz) is reached after about 20 min.

A control measurement, where first 5 μl anti-EBV antibody at a concentration of about 1 mg ml^{-1} were added, shows a frequency drop of about 300 Hz (Fig. 5), which probably results from unspecific (adsorptive) binding sites still remaining. The theoretical value for unspecific binding in Table 1 is zero.

After adding specific antibodies (again 5 μl at a concentration of about 1 mg ml^{-1}) a frequency shift of about 800 Hz could be measured (time constant about 35 min). A comparison of these measurements shows that the contribution of unspecific adsorption to the measured signal of Fig. 4 still remains, but is far below the total frequency shift. Nevertheless these preliminary results show clearly that a direct monitoring of antigen-antibody binding in liquids is feasible.

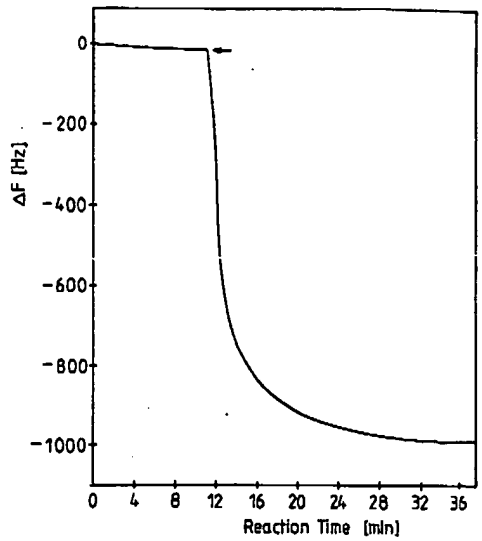

Fig. 4. Response for the reaction of anti-HIV antibody (dilution 1:1000) with adsorbed HIV peptide.

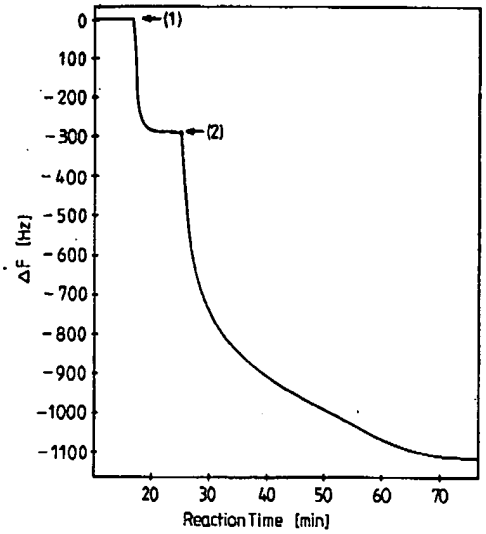

Fig. 5. Response for the unspecific adsorption of anti-EBV antibody to HIV peptide covered quartz (1) and the specific reaction of anti-HIV antibody (2).

DISCUSSION AND CONCLUSIONS

Using the measurement system described the piezoelectric crystal can be operated continuously in liquids at a frequency of 20 MHz.

The advantage of the demonstrated method of performing the experiments is the comparatively simple measurement set-up without pumps and valves for the flow-through cell. This also means much greater sample volumes compared to those

in flow-through cells. The experiments show the potential suitability of this measurement system as an analytical method for the observation of binding reactions *in situ*. Furthermore, they provide the basic tools for an immunosensor for the detection of anti-HIV antibodies in human serum.

Table 1 summarizes the results of the experiments, which show that adsorbed proteins form a stable binding to the gold surface of the oscillating quartz electrode. Thus the immobilization of a receptor layer (here antigen) can be performed by mere adsorption and, provided that the molecular weight of the receptor molecule is high enough, the efficiency of immobilization can be controlled by the sensor itself. The high standard deviation of the measurements indicates a poor reproducibility of the adsorption conditions. This means improvement of the sensor surface treatment is essential.

The specific binding of monoclonal antibodies to adsorbed HIV peptides could be monitored online. According to control experiments, however, an additional effect which amounts to about one third of the reaction is produced by unspecific binding. Therefore the saturation of unspecific binding sites after coating the quartz with HIV peptide has to be optimized and, in addition, the measurement has to be performed in a differential mode with two piezoelectric sensors. This differential set-up should also reduce the influence of temperature variations on the frequency shift.

The comparison of the measured data with the calculated data show that the Sauerbrey formula provides sufficient accuracy for estimations. However, more knowledge of the viscoelastic properties of bound protein monolayers would be helpful for more accurate calculations and for a deeper comprehension of interface and surface effects.

The surface properties and therefore the preparation of the surfaces are of decisive importance for the response values, the response times and the sensitivity. The experimental boundary conditions can be controlled more precisely when using a flow-through cell, a stirred solution and additional washing steps, with an optimized saturation of unspecific binding sites and with a differential measurement method.

The described method of carrying out experiments, however, possibly provides a simple disposable measurement system for a fast and easy control of blood serum. This kind of application does not have as strict demands concerning reversibility as well as long-term stability of resonant frequency and sensor coating as applications in fermentation control in bioreactors or in environmental observation. On the other hand, the costs of disposable sensor systems have to be low, which is also an argument for the relatively cheap quartz crystals.

ACKNOWLEDGEMENTS

The authors wish to thank the following: Dr Susanne Modrow for the preparation of HIV peptides (Max von Pettenkofer-Institut, München); Norbert Schertler, Michael Engelbrecht and Markus Tristl (IFT) for measurements and electronics; Elke Drath (IFT) for drawings; and BMFT for financial support.

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Article title	Characterization of immobilization methods for African swine fever virus protein and antibodies with a piezoelectric immunosensor
Article identifier	0956566398004204
Authors	Uttenthaler_E Kosslinger_C Drost_S
Journal title	Biosensors and Bioelectronics
ISSN	0956-5663
Publisher	Elsevier UK
Year of publication	1998
Volume	13
Issue	12
Supplement	0
Page range	1279-1286
Number of pages	8
User name	Adonis
Cost centre	
PCC	$22.00
Date and time	Tuesday, September 09, 2003 4:30:03 AM

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Biosensors & Bioelectronics 13 (1998) 1279–1286

BIOSENSORS & BIOELECTRONICS

Characterization of immobilization methods for African swine fever virus protein and antibodies with a piezoelectric immunosensor[1]

Erich Uttenthaler *, Conrad Kößlinger, Stephan Drost

Fraunhofer-Institute for Solid State Technology, Hansastrasse 27d, 80686 München, Germany

Received 16 February 1998; received in revised form 26 June 1998; accepted 30 June 1998

Abstract

A direct piezoelectric flow injection analysis immunoassay for the detection of African Swine Fever virus and antibodies is presented. The peptide-specific monoclonal antibody 18BG3 and the virus protein 73 were used for detection with a quartz crystal microbalance. Accumulation of the analyte on the surface of this mass-sensitive biosensor resulted in a shift of the resonant frequency. Highly selective receptor layers were applied on the sensing electrode of the quartz crystal for detection of the complementary analyte. Different immobilization methods proved to be appropriate for coating of the monoclonal antibody 18BG3. A quartz crystal covalently coated with the antibody 18BG3 detected virus protein VP73 samples more than 20 times and was stable for more than 30 days. The coating of virus protein was performed by physisorption. A sensor with a virus protein receptor layer detected antibody 18BG3 samples 10 times within one day. The sensor device was able to perform one measurement cycle including blocking and regeneration within 30 min. With the help of a suitable carrier liquid, measurements with serum samples were performed. The calibration curves for measurements in buffer and in serum could be determined and the detection limits for virus protein detection were 0.31 and 1 μg/ml, and for antibody detection 0.1 and 0.2 μg/ml, respectively.

Keywords: African swine fever; Immobilization; Immunosensor; Quartz crystal microbalance

1. Introduction

African Swine Fever (ASF) is an endemic disease in Spain and Portugal (Vieira, 1991). An extensive field survey in the endemic regions is necessary and diagnostic methods are needed for the recognition of the disease at an early stage of infection. A rapid, sensitive and specific technique for detection of this disease would help to minimize the dangers of epidemics (Wilkinson, 1991). Classical techniques for detection of the disease are the immunofluorescent test and the enzyme-linked immunosorbent assay (ELISA) (Terpstra, 1991). These laboratory tests use virus protein 73 (VP73) and a set of monoclonal antibodies for the detection of anti-ASF antibodies and ASF virus, respectively. VP73 is one of the major proteins of the ASF virus envelope with a molecular weight of 73 000 daltons and shows no variations under different genotypes of the ASF virus. Thus it provides a possibility for a common detection of different ASF strains and it appears to be an excellent antigen in classical tests (Hess, 1981; Wardley, 1983).

A direct immunosensor, the quartz crystal microbalance (QCM), also called a bulk acoustic wave (BAW) device, was integrated in a flow injection analysis (FIA) system. This system had, compared with classical immunological tests, the advantage of working continuously and monitoring binding of the analyte on line. Piezoelectric quartz crystals with an appropriate receptor layer were used as highly specific sensors. The resonant frequency of quartz crystals is influenced by the antigen–antibody reaction on the sensor surface due to the corresponding mass change (Sauerbrey, 1959). Therefore, the binding of the analyte can be detected directly based on the decrease of the resonant frequency of the quartz crystal. Physical properties of liquids, especially the vis-

* Corresponding author. Fax: + 49 (0)89 54759 100; E-mail: uttentha@ift.fhg.de

[1] This paper was presented at the Fifth World Congress on Biosensors, Berlin, Germany, 3–5 June 1998.

0956-5663/98/$ - see front matter
PII: S0956-5663(98)00089-X

cosity, have to be considered as side-effects for measurements in liquids (Kanazawa and Gordon, 1985). Contributions to the use of a QCM device for immunological applications have previously been published by Aberl et al. (1994); Kößlinger et al. (1994, 1995); Steegborn and Skládal (1997). The detection of infected pig sera with a QCM biosensor for classification is shown by Uttenthaler et al. (1998). The detection of African Swine Fever virus and antibodies with an optical and an amperometric biosensor is presented by Stiene and Bilitewski (1997); Vidal and Oliva (1997), respectively.

Fundamental to the QCM immunosensor is the sensing surface made up of antibodies or antigens. The requirements for an efficient receptor layer are a high surface density of functional molecules, the absence of non-specific binding, and a reliable stability (Ahluwalia et al., 1991). A variety of immobilization methods for proteins has been reported in the literature (Weetall, 1975; Hartmeier, 1986; Hermanson, 1992). The following methods were considered in this study: physisorption, coating with the streptavidin-biotin system, covalent coupling on a silanized surface and via a crosslinker.

The physical adsorption of protein molecules on the gold electrode of a quartz crystal is due to the fact that protein molecules adsorb strongly and irreversibly on a gold surface because of hydrophobic and thiol–gold interactions (Horisberger and Vauthey, 1984). Another non-covalent immobilization technique is the coating of biotinylated receptor molecules on a streptavidin layer (Livnah et al., 1993). Covalent coupling of receptor molecules on organic silane layers produces strong chemical bonds that impede the washing out of the receptor molecules in a flow system (Weetall, 1975). Covalent coupling on gold surfaces can also be performed by the help of a crosslinker molecule, e.g. dithiobis-succinimidyl propionate (DSP). The bifunctional crosslinker DSP chemisorbs rapidly to gold surfaces and the resulting support reacts quite efficiently with primary amine-containing ligands to form a stable peptide bond (Hermanson, 1992, p. 59).

In this publication, we focused on the establishment of a QCM immunoassay for detection of ASF antibodies and virus protein 73. We performed and compared different immobilization techniques for the monoclonal antibody 18BG3 and virus protein 73, which allow for regeneration and a long sensor lifetime. The antibody 18BG3 and virus protein 73 samples were also used for the evaluation of the receptor layers. With the appropriate coatings the calibration curves for the detection of monoclonal and polyclonal antibodies and virus protein 73 could be determined.

2. Experimental

2.1. The quartz crystal microbalance and the flow injection analysis system

The QCM sensor is a thin plate of AT-cut quartz crystal with gold electrodes on both sides. Two types of quartz crystals with fundamental resonant frequencies of 10 and 20 MHz and a diameter of 8 mm, delivered by Quarzkeramik GmbH, Stockdorf, Germany, were used. These resonant frequencies correspond to a thickness of the quartz crystals of 164 and 82 μm, respectively. Due to the piezoelectric properties of quartz, the mechanical resonance of the quartz crystals can be electrically excited. The change of the surface mass density, e.g. by specific adsorption, leads to a measurable shift of the resonant frequency of the quartz crystal. Sauerbrey (1959) calculated the frequency shift under the condition of small changes of surface mass density and resonant frequency. The frequency shift is given by $\Delta f = S \cdot \Delta m$ where Δf is the change of the resonant frequency (Hz), S is the mass sensitivity of the quartz crystal (S^{-1} = 1.1 ng/(Hz·cm^2) and S^{-1} = 4.3 ng/(Hz·cm^2) for 20 and 10 MHz quartz crystals, respectively) and Δm is the change of surface mass density (ng/cm^2). The formula indicates that 20 MHz quartz crystals are theoretically four times more sensitive than 10 MHz quartz crystals.

The apparatus used in this study has previously been described (Kößlinger et al., 1994). It consists of a fluid circuit with a flow-through cell, an injection valve and a pump. The electronic components comprise the oscillator circuit, the frequency counter and a personal computer. The quartz crystals were fixed to a synthetic carrier by a silicone adhesive and after immobilization of the sensitive layer they are mounted in the flow-through cell. The gold electrodes were connected to the oscillator circuit by gold bonding. Short-circuits between the quartz electrodes are avoided by exposing only one quartz electrode to the test liquid. A self-developed piston pump guaranteed a continuous flow of buffer liquid with small fluctuations and a flow rate of 30 μl/min through the flow-through cell. A high-pressure liquid chromatography (HPLC) injection valve with a 100 μl sample loop was employed for injection of the samples. A self developed 60 MHz frequency counter was used for recording of the resonant frequency. Computer control was performed by an IBM compatible 80486 personal computer via an I/O bus system.

2.2. Materials

18BG3 antibodies, biotinylated 18BG3 antibodies and lyophilized virus protein 73 were obtained from Ingenasa (Madrid, Spain). Lyophilized polyclonal antibodies against VP73 were supplied by the Gulbenkian Institute (Oeiras, Portugal). Acetone, casein, bovine serum albu-

min (BSA), 3,3'-dithio-bis(propionic acid n-hydroxysuccinimide ester) (DSP), glycerol, streptavidin,, TWEEN 20, negative pig serum and a biotinylation kit were delivered by Sigma (Deisenhofen, Germany). 3-(2-Aminoethylamino)-propyltrimetoxysilane (AAMS) was obtained from Fluka (Deisenhofen, Germany), glutardialdehyde by Polysciences, Inc. (Warrington, USA) and the regeneration buffer 1 M boric acid/KCl-NaOH (pH 11) by Merck (Darmstadt, Germany). The phosphate buffered saline (PBS) was 138 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 in distilled water (pH 7.4).

2.3. Coating procedures

Preceding the incubation procedures, the quartz crystals were cleaned by rinsing three times with acetone and distilled water. All subsequent incubation steps took place in a wet chamber. After immobilization the quartz crystals were rinsed with PBS and mounted in the flow-through cell and immediately used for the experiments. The following coating procedures for the monoclonal antibody 18BG3 and virus protein 73 were used in this study.

2.3.1. Physical adsorption (physisorption)

Incubation of 18BG3 (1 mg/ml PBS) on the cleaned gold electrode at 4°C overnight. Incubation of VP73 (1 mg/ml PBS + 0.1% sodium acide) on the cleaned gold electrode at room temperature for 16 h, 3 days, 4 days and 7 days.

2.3.2. Streptavidin-biotin system

Incubation of streptavidin (1 mg/ml PBS) on the quartz crystal at room temperature for 1 h. Immediately afterwards the quartz crystal was rinsed with PBS and incubated with biotinylated 18BG3 (1 mg/ml PBS) or biotinylated VP73 (1 mg/ml PBS) at 4°C overnight. Biotinylation of VP73 was performed as indicated in the biotinylation kit.

2.3.3. Covalent coupling on a silanized transducer surface

The quartz crystal plates were coated with silicon nitride by plasma vapour deposition. Subsequent silanization was performed with AAMsilane at 100°C under reflux for 7 h. They were cleaned by rinsing with acetone and water. After silanization the sensors were stored dried until needed. Then they were fixed on the carrier and incubated with glutaraldehyde (2.5% v/v in PBS) at room temperature for 1 h. After rinsing with distilled water, 18BG3 (1 mg/ml PBS) or VP73 (1 mg/ml PBS) was incubated on the sensor at 4°C overnight.

2.3.4. Covalent coupling via the crosslinker DSP

DSP (0.01 M in water-free acetone) was incubated on the gold electrodes of the sensor for 10 min at room temperature. Immediately afterwards the quartz crystal was rinsed with ethanol, dried and incubated with 18BG3 (1 mg/ml in PBS) or VP73 (1 mg/ml PBS) at 4°C overnight.

2.4. Measurement procedure

The coated quartz crystals in the flow-through system were under continuous flow of the carrier buffer until the frequency had stabilized under flow conditions (30 μl/min). Drying of the coated transducers was avoided.

The carrier buffer depended on the type of measurement:

- For measurements with *buffer samples*, PBS was used as the carrier buffer. The blocking reagents BSA (1 mg/ml PBS) or casein (1 mg/ml PBS) were applied to the sensor via the injection valve prior to the samples or directly mixed to the carrier liquid. Samples were diluted in the carrier liquid.
- For measurements with *pig serum samples*, a buffer composition of PBS with 4% (w/v) glycerol, 0.1% (v/v) TWEEN and 0.25% (w/v) casein proved to be appropriate (Uttenthaler et al., 1998). In this case it was favourable to add the blocking reagent casein directly to the carrier liquid. Samples were diluted in the carrier liquid (10 parts) and negative pig serum (1 part).

After blocking different dilutions of the samples were injected onto a coated sensor. In the case of a positive sensor signal, i.e. binding of the analyte, the sensor was regenerated in order to obtain free binding sites again. Regeneration was performed by an alkaline pH shift with the regeneration buffer boric acid/KCl-NaOH (pH 12.3). The sample volume was 100 μl for all experiments and the time between two consecutive measurements was about 10 min, depending on the dispersion of the sample peak.

Fig. 1 shows an example for a measurement curve of a QCM sensor. The binding of two concentrations of the analyte and subsequent regeneration is depicted.

2.5. Sensor signal and curve fitting

The ratio of the frequency decrease and the frequency noise, i.e. the signal-to-noise ratio, was used for a better comparability between signals of 10 and 20 MHz quartz crystals. The noise was determined as three times the standard deviation in a 20 min period of the frequency signal. The noise was 1.2 and 3.1 Hz for 10 and 20 MHz quartz crystals, respectively. Since the frequency decrease and the frequency noise are both dependent on the resonant frequency, the signal-to-noise ratio is inde-

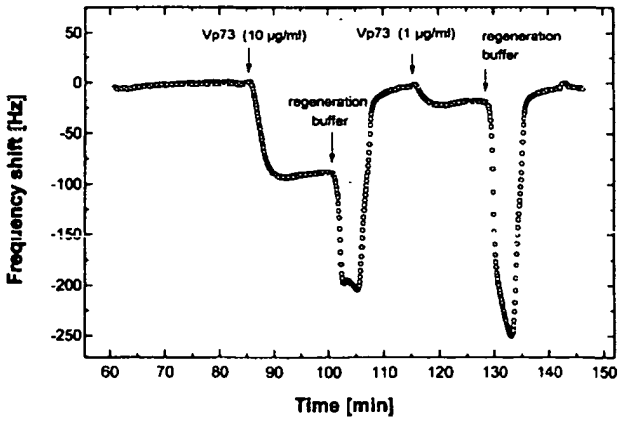

Fig. 1. Resonant frequency of a streptavidin-coated 10 MHz QCM sensor. On this sensor biotinylated antibodies were coupled on a streptavidin layer and virus protein 73 as a test sample was detected in a concentration of 10 and 1 µg/ml. Regeneration was performed by an alkaline pH shift.

pendent of the resonant frequency, as shown in the results and the discussion.

The calibration curves were determined by a fit of the measurement values of the dilution series to a sigmoid curve represented by the logistic function: $y = A\cdot(1 - (1 + (\frac{x}{x_0})^{p})^{-1})$ where A (Hz) is the saturation value, i.e. maximal response, x_0 (µg/ml) is the test middle point, i.e. the concentration for half-maximal response, p is power (corresponds to the slope of the curve). The fitting was performed with the scientific software 'Origin 4.0' (Microcal, Northampton, USA).

The detection limit is defined as the concentration of the analyte with a signal corresponding to three times the standard deviation of the frequency signal (frequency noise).

3. Results

3.1. Immobilization of the monoclonal antibody 18BG3

The monoclonal antibody 18BG3 was immobilized via physical adsorption, the streptavidin-biotin system, a silanized transducer surface and the crosslinker DSP as described in the experimental section.

The comparison of the different techniques was performed regarding the following properties: the signal-to-noise ratio after application of a test sample, the lifetime of the sensor and the number of performed regenerations during the lifetime. Moreover, the expenditure of time and costs of the different methods was assessed. Virus protein 73 with a concentration of 10 µg/ml was used as a test sample for all the differently coated quartz crystals. The lifetime of the sensors is given as the period of time during which reasonable results, i.e. greater than 25% of the value of the first positive sensor signal can be obtained. The lifetime is strongly related to the number of regenerations, therefore, the number of regenerations was also taken into account for the comparison.

Table 1 gives an overview of the results. The signal-to-noise ratio of the differently coated quartz crystals is in the range of 20 to 31, which is sufficient for immunosensing. The test sample caused almost the same frequency shifts on streptavidin and DSP-coated sensors. Antibodies immobilized by adsorption or via streptavidin had only a short lifetime of one measurement or 2 days, respectively. Compared with these methods, sensors covalently coated by silanization or by DSP had a much longer lifetime of 26 and 36 days, respectively. Covalently coated sensors could be regenerated for more than 20 times, whereas regeneration of a streptavidin-coupled sensor could be performed only seven times and regeneration of an adsorption-coupled sensor was not possible at all.

A comparison showed that binding of a VP73 sample on a streptavidin-18BG3 antibody-coated sensor yielded 63 Hz on a 20 MHz quartz crystal and 21 Hz on a 10 MHz quartz crystal. This gives a ratio of the signals for 20 and 10 MHz quartz crystals of 3, whereas the frequency noise ratio was shown to be 2.6.

The assessment of the experimental expenditure which combines factors like cost, work and preparation time showed that pure adsorption of antibodies needs only a very low experimental expenditure (Table 1). Streptavidin-activation is costly due to the facts that biotinylation of the antibodies is necessary and streptavidin is an expensive protein. Immobilization by silanization is time consuming and expensive, because technological steps like plasma vapour deposition and silanization of the quartz crystals are necessary. The experimental expenditure for the DSP activation is low, because this procedure can be carried out in only two steps: the binding of the crosslinker and subsequent incubation with the antibodies.

The time profile of the deactivation of a DSP activated sensor is seen in Fig. 2. An exponential curve fits the decrease in sensitivity over 15 cycles in a period of 36 days. Also for the other immobilization techniques an exponential deactivation down to approximately 25% of the first signal was observed during the lifetime of the sensor.

3.2. Immobilization of virus protein 73

For the coating of virus protein 73 the same techniques as for the antibody immobilization were applied, and the results are presented in Table 2. The signal-to-noise ratio after application of 20 µg/ml 18BG3 antibodies, the number of performed regenerations and the sensor lifetime were determined. The results show that

Table 1
Assessment of the immobilization techniques for the antibody 18BG3

Immobilization method	Resonant frequency (MHz)	Signal/noise ± SD	Regenerations (number)	Lifetime (days)	Experimental expenditure
Adsorption	20	29 (n = 1)	0	1	Very low
Streptavidin	10	31 ± 16 (n = 7)	7	2	High
Silanization	20	20 ± 13 (n = 27)	36	26	Very high
DSP	10	27 ± 14 (n = 15)	23	36	Low

The different methods were compared regarding the signal-to-noise ratio after application of a test sample (10 μg/ml VP73), the number of regenerations performed with the sensor and the lifetime of the sensor. One representative sensor for each technique was used. The experimental expenditure in time and cost is assessed.

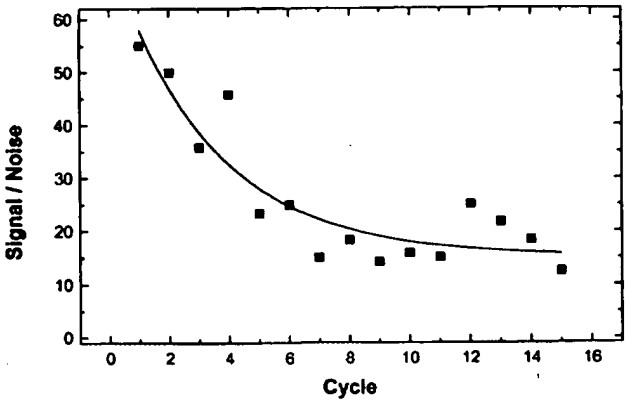

Fig. 2. Deactivation profile for the binding of virus protein 73. Antibodies 18BG3 are immobilized on a DSP-activated 10 MHz quartz crystal. Fifteen cycles of blocking, application of the test sample (10 μg/ml VP73) and regeneration were performed in a period of 36 days. An exponential curve fits the decrease of the signal-to-noise ratio.

the frequency shift of adsorptively coated QCM sensors increased with longer adsorption times. The streptavidin, silanization and crosslinker technique yielded only sensor signals much smaller than for adsorptively coated sensors. The sensor lifetime was limited to one day for all the methods. On a quartz crystal adsorptively incubated for 16 h three regenerations could be performed. A longer incubation period also increases the number of regenerations up to 10 in the case of physisorption for 4 days. The other methods allowed the regeneration of the sensors two times at the most.

A comparison showed that binding of a 18BG3 antibody sample on an adsorptively VP73-coated sensor yielded 165 and 60 Hz on a 20 and a 10 MHz quartz crystal, respectively. This gives a ratio of the signals for 20 and 10 MHz quartz crystals of 2.75.

The expenditure of the different methods has already been described in the preceding section, but because of the long preparation time of the sensors, the experimental expenditure was judged 'low' and not 'very low' as was done for incubation overnight.

3.3. Calibration curves

The calibration curves for detection of virus protein 73, the monoclonal antibody 18BG3 and polyclonal antibodies in buffer and in diluted serum were determined.

The sensors for detection of VP73 in buffer and in diluted pig serum were DSP activated and coated with 18BG3 antibodies. The standard curves are depicted in Fig. 3. The half maximal response of the sensors is at 18 and 5 μg/ml for measurements in buffer and in diluted serum, respectively. The detection limit was found to be 0.31 and 1 μg/ml in buffer and in diluted serum, respectively (Table 3).

Table 2
Assessment of the immobilization techniques for virus protein 73

Immobilization method	Resonant frequency (MHz)	Signal/noise ± SD	Regenerations (number)	Lifetime (days)	Experimental expenditure
Adsorption (16 h)	10	84 ± 1 (n = 3)	3	1	Very low
Adsorption (4 days)	10	213 ± 10 (n = 3)	10	1	Low
Adsorption (7 days)	10	254 ± 41 (n = 3)	5	1	Low
Streptavidin	10	6 ± 5 (n = 4)	1	1	High
Silanization	10	40 ± 3 (n = 2)	2	1	Very high
DSP	10	63 (n = 1)	0	1	Low

The different methods were compared regarding the signal-to-noise ratio after the application of 20 μg/ml 18BG3 antibodies, the number of regenerations performed with the sensor and the lifetime of the sensor. One representative sensor for each technique was used. The experimental expenditure in time and cost is assessed.

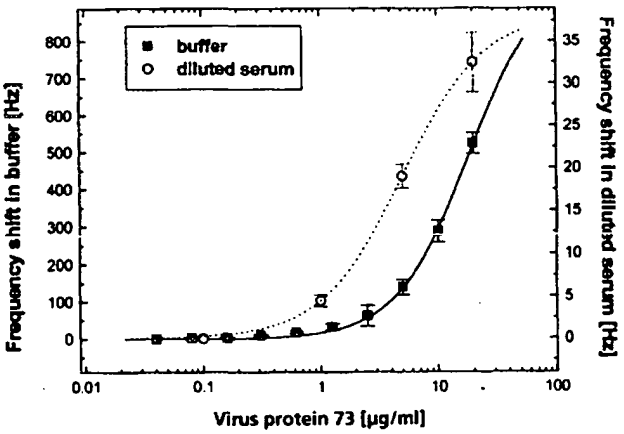

Fig. 3. Calibration curve for the detection of virus protein VP73 in buffer and in diluted pig serum. For the measurements in buffer and in diluted serum a 20 MHz quartz crystal and a 10 MHz quartz crystal, respectively, were used. At least two measurements were made for each concentration.

For the detection of monoclonal 18BG3 antibodies the quartz crystals were adsorptively coated with VP73 for 4 days. Fig. 4 shows the two calibration curves for the measurements in buffer and in diluted serum. As depicted in Table 3 the half maximal response could be determined to be at 14 and 30 µg/ml and the detection limit 0.1 and 0.2 µg/ml in buffer and in diluted serum, respectively.

Using the experience of the preceding experiments polyclonal antibodies were titrated and detected with a VP73-coated QCM sensor similar to the detection of monoclonal antibodies. Two calibration curves for the measurements in buffer and in diluted sera are depicted in Fig. 5. The detection limit is a dilution of 1 to 2000 in the carrier liquid and the half maximal response was at 1 to 280 and 1 to 200 for measurements in buffer and in diluted sera, respectively (Table 3).

4. Discussion and conclusion

The comparison of different immobilization methods revealed useful properties of each technique. This helped us to optimize the immobilization of 18BG3 antibodies and virus protein 73.

The signal-to-noise ratio after application of a VP73 test sample on a streptavidin-18BG3-coated sensor was

Table 3
Detection limit and test middle points of the measurements

Measurement	Detection limit	Test middle point
VP73 in PBS (µg/ml)	0.31	18
VP73 in diluted pig serum (µg/ml)	1	5
18BG3 in PBS (µg/ml)	0.1	14
18BG3 in diluted pig serum (µg/ml)	0.2	30
Polyclonal antibodies in PBS (dilution)	1 to 2000	1 to 280
Polyclonal antibodies in diluted pig serum (dilution)	1 to 2000	1 to 200

Summary of the experimental results by indicating the detection limits and the test middle points at half maximal response for detection of the analyte in PBS buffer or diluted serum.

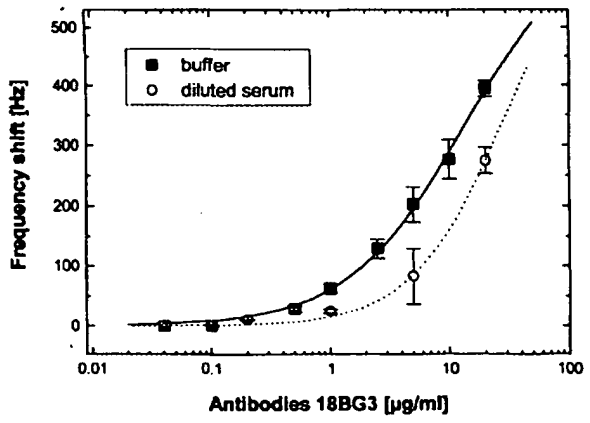

Fig. 4. Calibration curve for the detection of the monoclonal antibody 18BG3 in buffer and in diluted pig serum. Two 20 MHz quartz crystals were used for the measurements in buffer and two 10 MHz quartz crystals were used for the measurements in diluted serum. For every concentration one measurement on each quartz crystal was performed.

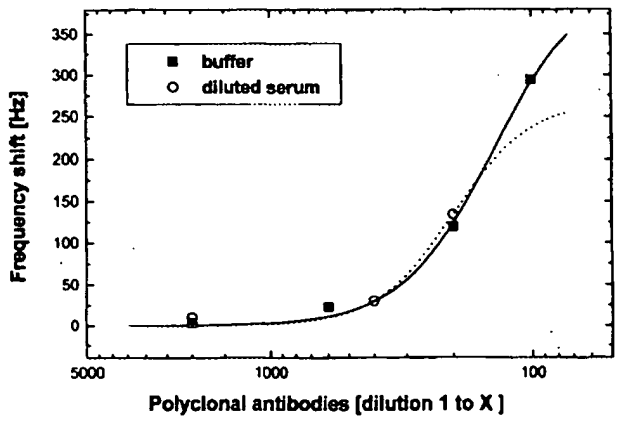

Fig. 5. Calibration curve for the detection of polyclonal antibodies in buffer and in diluted pig serum. A 20 MHz quartz crystal was used for the titration measurement in buffer and a 10 MHz quartz crystal for the titration series in diluted serum.

determined to be 20.3 and 17.5 for a 20 and a 10 MHz quartz crystal, respectively. Similarly, the ratio for detection of an 18BG3 sample with an adsorptively coated sensor was 53 and 50 for a 20 and a 10 MHz quartz crystal, respectively. The differences of less than 15% are within the usual signal fluctuation of identically coated quartz crystals. For this reason the signal-to-noise ratio proved to be independent of the resonant frequency and, therefore, it allowed comparison of the measurement signals of 10 and 20 MHz quartz crystals.

The monoclonal mouse antibody 18BG3 was shown to be an ideal tool for studying the different immobilization techniques. In summary, the results showed that the adsorption, streptavidin and the crosslinker technique resulted in appropriate sensor signals, but only the crosslinker technique also provided a long sensor lifetime. Apparently, covalent coupling increases the stability against degradation during the regeneration process and results in a reduced washing out. Antibody immobilization by pure adsorption in our case was only stable for one measurement. Since our need was a reusable sensor, this simple method was not suitable for our application. The streptavidin-biotin method showed good performance for seven measurements. Obviously, the streptavidin-antibody complex was more stable than the antibody 18BG3 layer alone regarding the immobilization.

During the lifetime the sensitivity continuously decreased to at least 25% of the first signal. This explains the large standard deviation given in Table 1 for the signal-to-noise ratio, where the mean value of all measurements with one sensor is calculated. Moreover, this investigation intended to examine qualitatively the lifetime of the sensors for the comparison of the different techniques and not to minimize the standard deviation. However, in the measurements for determining the calibration curves, we tried to minimize the standard deviation by using newly coated quartz crystals for each experiment.

The time profile in Fig. 2 showed that the DSP sensor is deactivated exponentially. A similar deactivation is seen for the other immobilization techniques. One main reason for this deactivation is an incomplete regeneration, which can be seen sometimes with the QCM device. Because of this the sensor loses binding capacity. Further investigations should help to optimize the regeneration process.

For immobilization of virus protein 73 most of the techniques used for antibody immobilization were not suitable, because of poor results. The only practicable immobilization technique for VP73 was the adsorptive coating of VP73 on the gold electrode of the quartz crystal. With this technique we obtained a sensor for about 10 measurements with subsequent regeneration. The sensor lifetime is limited to one day, probably because of a continuous deactivation of the adsorptively bound receptor molecules. The results showed that an optimum in the incubation period is reached after 4 days of incubation. This long incubation period of VP73 is unusual, compared with other proteins, where some hours are sufficient. Probably the structure of VP73 is influenced by immobilization which results in reduced biological activity. After only a few days VP73 forms a stable structure on the sensor. Vidal and Oliva (1997) reported a similar procedure, the adsorptive coating on polymer membranes. In that case, regenerations were not performed and new membranes were used for each experiment. The binding of biotinylated VP73 on a streptavidin-coated surface resulted in a non-regeneratable sensor with a small sensor signal. We assume that the biotinylation of VP73 failed due to the instability of this peptide. Stiene and Bilitewski (1997) succeeded in coating of biotinylated VP73 on streptavidin-coated glass beads. Also in that study a rapid loss of sensitivity of the VP73 receptor layer is reported and, therefore, each measurement was performed on a new streptavidin-VP73 layer.

The calibration curves indicate that a QCM sensor integrated in a FIA system is appropriate for the detection of African Swine Fever virus protein and antibodies. Detection of the analyte in diluted pig serum was possible but the performance was restricted due to the strong non-specific binding of serum proteins (Uttenthaler et al., 1998). The strong blocking buffer applied against non-specific binding increased the noise of the sensor signal. Therefore, the detection limit for measurements in serum was higher than for measurements in buffer, except for the detection of polyclonal antibodies (Table 2). In this case it is possible that the polyclonal antibodies were stabilized in the serum solution.

The calibration curve for the detection of VP73 in buffer indicates that the maximal sensor signal is approximately 800 Hz for a completely covered 20 MHz sensor (Fig. 3). This value corresponds to the saturation value of the fitting data. With the help of the Sauerbrey formula the resulting surface density of VP73 is calculated to be approximately 0.89 $\mu g/cm^2$ or $1.22 \cdot 10^{-11}$ mol/cm^2. For detection of VP73 in serum we obtained a surface density of virus protein of only $0.24 \cdot 10^{-11}$ mol/cm^2, which is only 20% of the surface coverage for the measurements in buffer. Although, different quartz crystals with probably slightly different receptor layer densities were used for the measurements, it is likely that the main difference in the surface density for measurements in buffer and in serum must be attributed to the strong blocking of specific binding sites with serum proteins. Furthermore, we assume that every antibody in the receptor layer bound two virus protein molecules. Therefore, the surface density of antibodies on the quartz crystal can be estimated to $0.61 \cdot 10^{-11}$ mol/cm^2 or 0.92 $\mu g/cm^2$. This antibody density is in the same range as the value given by Ahluwalia et al. (1991). In

that study the density of covalently coated antibodies was determined to be 0.5 $\mu g/cm^2$ by ellipsometry.

Two sensor devices which worked on ASF virus protein and antibodies are reported in the literature. The electrochemical sensor presented by Stiene and Bilitewski (1997) has a narrow dynamic detection range with a lower detection limit of 1 ng/ml for detection of antibodies. The optical immunosensor presented by Vidal and Oliva (1997) reaches a detection limit of 50 ng/ml in a competitive immunoassay. Despite a higher detection limit the QCM sensor has the advantage of being a direct immunosensor. An enzymatic reaction or the use of labelled antibodies as for the electrochemical and optical sensor devices is not necessary, since the binding of the analyte is recognized directly by the QCM. This real time performance also allows the study of the kinetics of immunoreactions and provides a possibility for quick measurements. Moreover, the whole QCM biosensor device can be reduced to a portable size for on-field diagnosis and allows a high degree of automatization. The knowledge in the immobilization of proteins on a quartz crystal biosensor facilitates the modification of the QCM sensor for the detection of other analytes. It is shown to be a flexible tool for all direct immunoassays.

Acknowledgements

This work was part of a European interlaboratory project and was supported by the European Community, project AIR3-CT92-0665.

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Development of a piezoelectric immunosensor for the detection of *Salmonella typhimurium*

E. Prusak-Sochaczewski and J. H. T. Luong

National Research Council of Canada, Biotechnology Research Institute, Montreal, Quebec, Canada

G. G. Guilbault

Department of Chemistry, Universit of New Orleans, New Orleans, LA

A piezoelectric biosensor has been developed for the detection of Salmonella typhimurium. *The antibody to* Salmonella *was immobilized on the crystal by various immobilization procedures. The best result was obtained when antibody was immobilized on the crystal precoated with a thin layer of polyethyleneimine. The response of the coated crystal for* S. typhimurium *in a microbial suspension was in the range of 10^5 to 10^9 cells ml^{-1}. The time required for a complete interaction between the crystal and the cells appeared to depend upon the cell concentration of the analyzed sample. The antibody-bound crystal lost no activity over 4 days at 4°C and it could be reused for 6–8 consecutive assays.*

Keywords: Piezoelectric crystal; antibody; *Salmonella*; immunosensor

Introduction

A rapid and reliable screening of *Salmonella* contamination in foodstuffs is very important to the food industry. Although many rapid procedures have been proposed for *Salmonella* detection over the years,[1,2] enzyme immunosorbent assays (ELISA) have gained the greatest acceptance.[3–5] Recently, considerable attention has been focused on the improvement of ELISA protocols to obtain sensitive and rapid assays[6–8] and on the production of highly specific monoclonal and polyclonal antibodies capable of detecting all *Salmonella* strains.[5,9]

A specific binding between antibody and antigen has been exploited in immunobiosensor construction by immobilizing antibody to a suitable transducer. Immunobiosensors based on electrochemical detection have been demonstrated for determination of proteins. Since many of the immunoreactions do not involve monitorable electroactive products or reactants, enzyme such as peroxidase is commonly linked to either antibody or antigen to produce the desired signal. Recently, other types of transducer have been used together with antibody/antigen to construct a sensitive and small immunobiosensor, notably the work of Karube and co-workers[10,11] who used a piezoelectric crystal system to assay *Candida albicans* in solution. Piezoelectric crystals are pieces of oscillating quartz on which is an adsorbent that selectively interacts with the analyte.[12] Adsorption of the analyte increases the mass of the crystal and decreases proportionally its basic frequency of oscillation, which is generally 9 or 14 MHz. The limit of detection of this technique is estimated to be about 10^{-12} g.[12] The first attempt to construct piezoelectric biosensors dates back to 1972 when Shons *et al.*[13] attached a layer of antigen to the surface of a 9-MHz crystal and used it to measure the amount of specific antibody in a liquid sample directly. To date, most of the previous analytical work with such detectors has centered on the use of inorganic or organic coatings for gaseous environmental pollutants such as HCl, isocyanates, CO, NO_x, and SO_2.[14] A review of this field is available from the literature.[14]

In this study, antibody t *Salmonella* was immobilized on the surface of a quartz crystal which then

Address reprint requests to Dr. H. Luong at the National Research Council of Canada, Biotechnology Research Institute, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2
Received 14 November 1988; revised 18 April 1989

selectively interacted with *Salmonella typhimurium*, producing a change in resonant frequency. The sensitivity, reliability, and reusability of the piezoelectric crystal were als investigated and are discussed in detail.

Materials and methods

Materials

Anti-*Salmonella* antibody (goat affinity purified to *Salmonella* common structural antigen) was purchased from Mandel Scientific Company Ltd. (Canada). *Salmonella typhimurium* LT2 (SA 1355 virulent strain) and *Escherichia coli* JM 83 strain were obtained from the National Research Council of Canada, Ottawa. The bacterial stock cultures were maintained on 1.5% nutrient broth agar (Difco) slants at 4°C. The bacteria were grown on the nutrient broth at 37°C. The viable cell count was used for determination of bacterial cell concentration. Decimal dilutions of bacteria were prepared in peptone water (Difco) and counts were done using pour plates of the nutrient agar incubated at 37°C for 2 days. Each developed colony was assumed to have grown from one viable cell.

Equipment

The piezoelectric crystal consisted of a 15 × 0.2-mm quartz wafer which was placed between 10-mm gold electrodes and mounted in a ceramic holder with a plug (International Crystal Mfg. Co., Oklahoma, USA). The system was similar to that shown by Shons *et al.*[13] The crystals were AT-cuts (referred to the angle of rotation) with a 9-MHz resonant frequency. An identical crystal was used as reference to correct for temperature fluctuations and other interferences. The frequency difference between the piezoelectric crystals was monitored by a frequency counter (model PZ-101, Universal Sensors, New Orleans, USA).

Immobilization procedures

Since all reactions on the crystal surface required dipping in reagent solutions, antibody to *Salmonella* was immobilized on both quartz and gold parts of the crystal.[13] Four different immobilization procedures were attempted for depositing antibody on the crystal surface.

Immobilization via a thin polymer layer. Three microliters of a methanol solution containing 2% polyethyleneimine (Aldrich) was dispersed on the surface of the electrode and the crystal was air dried. The crystal was then placed in a chamber containing 2.5% glutaraldehyde (pH 7) for 30 min. Anti-*Salmonella* antibody was immobilized on the crystal by adding 5 μl of the antibody (concentration 3 mg ml^{-1}) at each side of the crystal. Incubation was carried out in a wet chamber containing deionized water for 1 h. The crystal was then immersed into a solution containing 0.1 M glycine in 20 mM phosphate, pH 7.2, with 0.17 M NaCl buffer (referred herein as PBS) to block all unreacted aldehyde groups. The crystal was subsequently rinsed with PBS, distilled water, and dried in air.

Immobilization via bi tin–avidin interaction. The crystal was first coated with a thin layer of polyethyleneimine and submerged into a glutaraldehyde solution as above. About 5 μl of avidin (1 mg ml^{-1}) was immobilized onto the polymer-coated crystal via the surface aldehyde groups. Anti-*Salmonella* antibody was biotinylated by the method described by Guesdon *et al.*[15] The biotinylated antibody was then reacted with avidin-coated crystal by depositing 5 μl of the biotinylated antibody (1 mg ml^{-1}) on each side of the crystal for 20 min. It is worth noting that one molecule of avidin can efficiently bind four molecules of biotin (association constant 10^{-15}–10^{-16} M). Avidin will act as a bridge between the biotinylated antibody and the polyethyleneimine-coated crystal. The use of biotin, a small molecule, to label antibody should not significantly alter the molecular size, i.e. the antigen-binding capacity of antibody. After washing with PBS and water, the resulting crystal was air dried.

Immobilization via glutaraldehyde cross-linking. A 3-μl solution (pH 7) containing bovine serum albumin (5 mg ml^{-1}) anti-*Salmonella* antibody (5 mg ml^{-1}) and glutaraldehyde (2.5%) was coated on each side of the crystal. Incubation was carried out in a wet chamber and in the air for 30 min and 2 h, respectively. The crystal was washed with water and the unreacted aldehyde groups were blocked with 0.1 M glycine in PBS. The crystal was washed again with PBS and water.

Immobilization via a thin silane layer. The crystal was dipped in a 5% solution of γ-aminopropyltriethoxy silane (Sigma) in dry benzene for 2 h at room temperature (23–25°C). The crystal was then vacuum dried at 50°C. The silanized crystal was activated by incubation for 1 h with 2.5% glutaraldehyde at pH 7. The anti-*Salmonella* antibody was then deposited on each side of the crystal by the method described above.

Measurement procedure. The frequency difference between the antibody-coated crystal and the reference crystal (uncoated) was first determined (ΔF_1). The coated crystal was then dipped in 2 ml of bacterial suspensions containing from 10^3 to 10^9 cells ml^{-1} for 30 min at room temperature. The bacterial suspensions were prepared from a bacterial stock (containing ~ 3 × 10^9 cells ml^{-1} *Salmonella* suspended in nutrient broth, pH 7) by decimal dilution using peptone, pH 7.2 solution. The crystal was rinsed with PBS and water and air dried. The amount of *Salmonella* cells adsorbed on the crystal surface was determined by counting the total cells remaining in the suspensions. The coated crystal was placed in the test cell and a new frequency difference was measured (ΔF_2). A shift in the frequency difference ($\Delta F = \Delta F_2 - \Delta F_1$) was related to the amount of *Salmonella typhimurium* cells ads rbed onto the crystal.

After each *Salmonella* measurement, the crystal was immersed into a s luti n containing 8 M urea t remove the adsorbed bacterial cells. After washing with PBS and water, the crystal was placed into the test cell to determine whether the adsorbed cells were completely diss ciated fron the antibody. If the bacterial cells were completely rem ved from the crystal, the determined frequency difference should return to its original value(ΔF_1).

Results and discussion

When the sensitivity and stability were taken into consideration, immobilization of antibody onto a surface of the crystal precoated with a thin layer of polyethyleneimine provided the most satisfactory result (*Table 1*). The thin layer attained by coating the crystal surface with polyethyleneimine (2%) was stable during the course of the measurement. The antibody-coated crystal could be reused several times and provided the most reproducible results. Immobilization via avidin–biotin interactions did not appear to improve the sensitivity of the detection procedure, since the ability of the avidin-coated crystal to adsorb *S. typhimurium* was almost identical to that of the former. Another disadvantage of the immobilization procedure via avidin–biotin interactions was noted. For repeated uses, it was necessary to submerge the antibody-bound crystal into a solution containing 8 M urea to remove adsorbed bacterial cells. Under such conditions, a partial desorption of the immobilized biotinylated antibody occurred. Dissociating agents such as 10 mM HCl, 0.2 M glycine-HCl pH 2.8, and 0.2 M ethanolamine pH 8 did not remove the adsorbed bacterial cells effectively.

A greater amount of the antibody could be immobilized on the crystal surface by direct immobilization via glutaraldehyde cross-linking of the antibody with bovine serum albumin. This method, however, resulted in a thick layer which was not stable during the pro l nged wet and dry procedure applied for the bacterial detection. The resulting antibody-coated crystal could also be used for only one *Salmonella* assay, since it could not withstand the regeneration step (8 M urea solution). Immobilization via a thin silane layer resulted in a very small amount of the bound antibody.

Table 1 Effect of different immobilization procedures on the sensitivity of the piezoelectric crystals (the number of *S. typhimurium* cells in the determined sample was 10^8 cells ml^{-1})

Method of antibody immobilization	Frequency change, ΔF, of the sensor due to *S. typhimurium* binding
Coupling with the polymer	330
Coating via biotin–avidin interactions	280
Direct glutaraldehyde cross-linking	530
Coupling with the silane	80

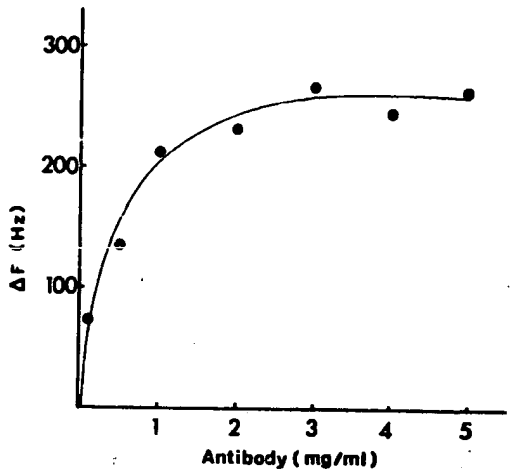

Figure 1 Effect of antibody concentration used for immobilization on the sensitivity of the piezoelectric immunosensor. The antibody-coated crystal was incubated with 10^8 *Salmonella* cells ml^{-1} for 30 min

Such behavior could be due to the fact that the untreated gold electrode of the crystal did not possess any hydroxyl groups necessary to react with γ-aminopropyltriethoxy silane. Consequently, a very small amount of the antibody was attached only to the quartz surface of the crystal. As a result of this finding, immobilization of the antibody via a thin polymer layer was used for subsequent studies.

A series of experiments was then carried out to determine the optimal concentration of antibody used for coating the crystal surface. As described in Materials and methods, after immobilizing the crystal with antibody to *Salmonella*, the crystal was dipped into the sample containing 10^8 cells ml^{-1} *Salmonella* for 30 min to allow reaction to occur between the immobilized antibody and the microorganism. The crystal was then extensively washed and dried in air. The resonant frequency was measured and plotted against the concentration of antibody used (*Figure 1*). Up to 3 mg ml^{-1}, the sensitivity of the biosensor increased with an increase in the antibody concentration used for immobilization. Beyond this level, the frequency change was no longer dependent upon the antibody level. The result obtained was not unexpected, since for a given immobilization surface, there will be a finite quantity of antibody that can be attached to the crystal surface. Based on this finding, the crystal surface coated with 3 mg ml^{-1} of antibody solution was used for subsequent studies.

Piezoimmuno crystals, surface coated with immobilized anti-*Salmonella* antibody, were then used to detect *Salmonella typhimurium*. The treated crystal was dipped in the microbial suspension to allow a binding between the crystal and the microbe. *Figure 2* shows that the elapsed time required for an interaction between the coated crystal and the microorganism appeared to depend on the microbial population of the

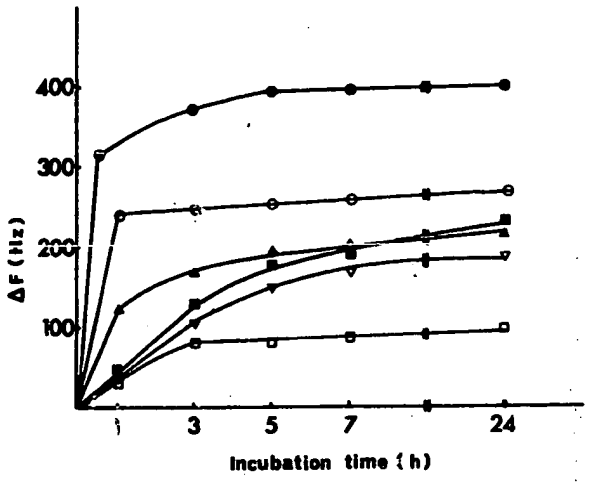

Figure 2 Effect of bacterial concentration and the required time for a complete binding between the crystal and microorganisms on the sensitivity of the piezoelectric crystal. Concentrations in cells ml^{-1}: (□) 10^3; (■) 10^5; (▽) 10^6; (▲) 10^7; (○) 10^8; (●) 10^9

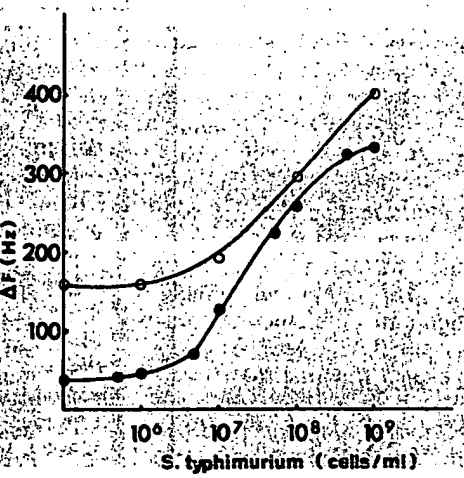

Figure 3 Relationship between *S. typhimurium* concentration and the resonant frequency of the piezocrystal coated with anti-*Salmonella* antibody. (●) 0.5 h of incubation *Salmonella* with the crystal; (○) 5.0 h of incubation *Salmonella* with the crystal

sample. For a low cell density sample (<10^5 cells ml^{-1}), about 3–5 h were required for a complete binding between the crystal and microorganism. However, at a higher cell density (>10^7 cells ml^{-1}), about 1–3 h were sufficient. Viable bacteria counts on nutrient agar plates showed that when crystals were incubated with a 2-ml suspension containing up to 10^5 cells ml^{-1} of *S. typhimurium* for 5 h, all cells were adsorbed onto the crystal surface (*Table 2*). Incubation of the crystals with samples containing higher cell populations indicated that the total cells that were adsorbed on the crystal surface were ca. 5×10^5–10^6 cells. It is worth noting that the piezoimmunosensor developed by Karube and Gotoh[10] was able to detect *C. albicans* in the range of 10^6–5×10^8 cells ml^{-1}.

Table 2 Determination of the number f *S. typhimurium* cells bound to the crystal for different *Salmonella* concentrations and a 5-h incubati n time

Number of cells c ntacted with a crystal	Number of bound cell determined by viable count	Frequency change ΔF, f the crystal
2×10^5	2×10^5	180
2×10^6	2×10^5	150
2×10^7	5×10^5	190
2×10^8	10^6	250
2×10^9	~5×10^6	390

The practical applicability of the piezoimmunosensor for the detection of *Salmonella*, therefore, was dependent on the interaction time between the crystal and the cells as well as the cell concentration of the sample (*Figure 3*). The piezocrystal sensor could detect as low as 10^5 *Salmonella* cells ml^{-1}. However, about 5 h were required for a complete binding between the crystal and the microbe. On the other hand, if the sample contained 10^7 cells ml^{-1} or more, only 0.5 h was required for dipping the crystal in the microbial suspension. In order to illustrate that change in the crystal oscillation was specific to *Salmonella* adsorption, samples of *E. coli* were reacted with the crystal. When the sensor was dipped into a microbial suspension containing 10^9 cells ml^{-1} of *E. coli*, the frequency difference was determined to be less than 40 Hz, indicating a minimum nonspecific interference. However, the polyethyleneimine-coated crystal without the immobilized antibody layer adsorbed an appreciable quantity of both *E. coli* and *S. typhimurium*, as re-

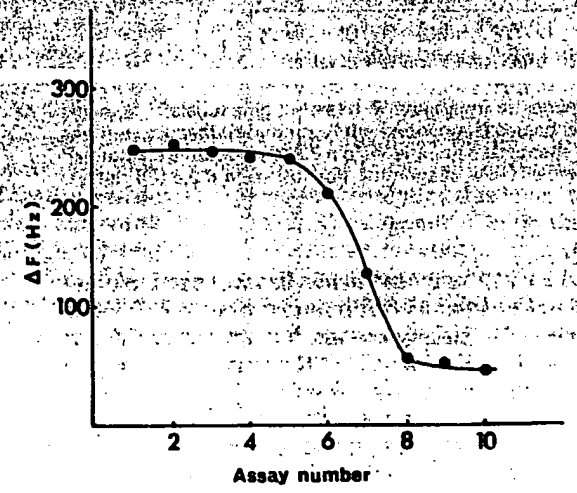

Figure 4 Detection sensitivity of the immunopiezo crystal for repeated uses. The crystal was incubated with *Salmonella* (10^8 cells ml^{-1}) for 30 min. Regeneration was performed by dipping the crystal in 8 M urea for 2 min and followed by washing with PBS and water

flected by a significant increase in the resonant frequency (70 Hz). This could be due to the fact that both *E. coli* and *S. typhimurium* were able to attach to the imine groups of the coated crystal. A steady-state response of the antibody-coated crystal was reached typically after 50 to 60 s compared with 10–15 s for the bare crystal. The coated crystal stored at 4°C showed no significant decrease in its response to *Salmonella* (data not shown). It is worth noting that (according to the supplier) the antibody used in this study is highly specific to *Salmonella* and it reacts with at least 87 *Salmonella* strains.

For repeated uses, the experimental data confirmed that bacteria could be removed from the crystal surface by washing the crystal with 8 M urea without denaturing the immobilized antibody layer. However, after six analyses, the sensitivity of the equipment declined very sharply (*Figure 4*). Apparently, the immobilized antibody was partly denatured and/or desorbed from the crystal surface.

In brief, this study has shown that antibody to *Salmonella* can be immobilized onto a piezocrystal surface to form an immunobiosensor for the detection of *Salmonella*. The technique is suitable and convenient for detection of *Salmonella* in foodstuffs. Obviously, extended experiments on food samples naturally contaminated with *Salmonella* are necessary, since such samples may contain interfering organisms other than *E. coli*, such as *Proteus*, *Citrobacter*, etc. Although the antibody used in this study is highly specific to *Salmonella* (reacts with at least 87 *Salmonella* strains), the behavior of the immunosensor in mixed cultures containing different organisms and food debris remains to be determined. In addition, a better method for antibody immobilization is yet to be developed to improve the sensitivity and the performance of the piezoimmunosensor.

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DERWENT-ACC-NO: 1989-123229
DERWENT-WEEK: 198917

TITLE: Coating piezoelectric quartz crystal with antibody-binding polymer - useful in immunoassay of antigen and determn. of affinity ligands

INVENTOR: LAURS, H; MULLERSCHU, D

PATENT-ASSIGNEE:

ASSIGNEE	CODE
MULLER-SCHULTE D	MULLI

PRIORITY-DATA: 1987DE-3733986 (October 8, 1987)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
DE 3733986 A	April 20, 1989		005	

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
DE 3733986A	October 8, 1987	1987DE-3733986	

INT-CL (IPC): B06B 1/06; C04B 41/63; G01N 29/00; G01N 33/53

ABSTRACTED-PUB-NO: DE 3733986A
BASIC-ABSTRACT:

A vibrating quartz crystal is coated with a synthetic polymer (I) which is able to bind, by adsorption or chemically, antibodies or other biologically active molecules to the quartz.

Specifically the coating is made with (I) which is insoluble in aq. soln. but soluble in organic solvent, particularly a copolymer in which one component has reactive side gps. able to couple to antibodies, etc..

USE/ADVANTAGE - The immobilised antibodies etc. are able to bind specific antigens or ligands, and the resulting change in mass detected by a change in the vibration frequency of the piezoelectric crystal. This provides for accurate immunoassays and affinity ligand determinations at the ng level. Problems of non-specific adsorption are avoided and no radioactive or enzyme labels are needed.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: COATING PIEZOELECTRIC QUARTZ CRYSTAL ANTIBODY BIND POLYMER USEFUL IMMUNOASSAY ANTIGEN DETERMINE AFFINITY LIGAND

DERWENT-CLASS: A96 B04 J04 P43 S03 V06

CPI-CODES: A12-B08; A12-E13; A12-E15; A12-V03C2; B04-B04C2; B04-B04C6; B04-C03B; B04-C03D; B04-D02; B11-C07A6; B12-K04A; J04-B01;

EPI-CODES: S03-E14H4; V06-K09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
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M314 M315 M320 M321 M323 M331 M332 M333 M342 M373
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